Background Review Document
Validation Study of the BG1Luc4E2 Estrogen Receptor (ER) Transcriptional Activation (TA) Test Method

Interagency Coordinating Committee on the Validation of Alternative Methods

National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods

National Institute of Environmental Health Sciences
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List of Abbreviations and Acronyms

ADME	Absorption, Distribution, Metabolism, and Excretion
AMP	Ammonium perchlorate
ANDRO	4-Androstenedione
API	Apigenin
APO	Apomorphine
AR	Androgen Receptor
ATP	Adenosine Triphosphate
ATZ	Atrazine
BBP	Butylbenzyl phthalate
BG1Luc ER TA	LUMI-CELL® BG-1Luc4E2 ER TA test method
BICAL	Bicalutamide
BPA	Bisphenol A
BPB	Bisphenol B
BRD	Background Review Document
DMSO	Dimethyl sulfoxide
CASRN	Chemical Abstracts Service Registry Number
CERI	Chemicals Evaluation and Research Institute, Japan
CHX	Cycloheximide
CHY	Chrysin
CLOM	Clomiphene citrate
CORT	Corticosterone
COU	Coumesterol
CUM	4-Cumylphenol
CV	Coefficient of Variation
CYP	Cyproterone acetate

DAI	Daidzein
DBA	Dibenzo[*a,h*]anthracene
DBP	Di-*n*-butyl phthalate
DDE	*p,p'*-DDE
DDT	*o,p'*-DDT
DEA	U.S. Drug Enforment Administration
DEHP	Diethylhexyl phthalate
DES	Diethylstilbestrol
DEX	Dexamethasone
DHT	5α-dihydrotestosterone
DIC	Dicofol
DRP	Detailed Review Paper
DMEM	Dulbecco's Modification of Eagle's Medium
DMSO	Dimethyl Sulfoxide
ECVAM	European Centre for the Validation of Alternative Methods
E1	17α-estradiol
E2	17β-estradiol
EC_{50}	Half–maximal effective concentration
ED_{50}	Effective dose of a drug that is pharmacologically effective for 50% of a population
ED	Endocrine Disruptor
EDSP	Endocrine Disruptor Screening Program
EDSTAC	EPA Endocrine Disruptor Screening and Advisory Committee
EDTA	Endocrine Disruptor Testing and Assessment (OECD)
EDWG	Endocrine Disruptor Working Group
EE	17α-Ethinyl Estradiol

EFM	Estrogen-Free Media
EPA	US Environmental Protection Agency
EPB	Ethyl paraben
ERE	Estrogen-Responsive Element
EST	Estrone
EPA	U.S. Environmental Protection Agency
EtOH	Ethanol
ER	Estrogen Receptor
ERE	Estrogen Responsive Element
FAST	Finasteride
FBS	Fetal Bovine Serum (Charcoal/Dextran Treated)
FDA	U.S. Food and Drug Administration
FEN	Fenarimol
FFDCA	Federal Food Drug and Cosmetic Act
FLA	Flavone
FLO	Fluoranthene
FLUT	Flutamide
FMES	Fluoxymestrone
FQPA	U.S. Food Quality Protection Act
FR	Federal Register
G418	Gentamycin
GEN	Genistein
GLP	Good Laboratory Practices
HEX	*meso*-hexestrol
HFLUT	Hydroxyflutamide
Hiyoshi	Hiyoshi Corporation

HPD	Haloperidol
hrER	Human Recombinant Estrogen Receptor
HSDB	The National Library of Medicine's Hazardous Substances Data Bank
I	Inadequate
IC	Inconclusive
IC_{50}	Concentration of the test substance that inhibits the reference estrogen response by 50%
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ILS	Integrated Laboratory Systems
ISO	International Organization for Standardization (ISO), Geneva, Switzerland
ISO 9000	An international quality management standard
JaCVAM	Japanese Center for the Validation of Alternative Methods
KCN	Ketoconazole
KEP	Kepone
KMP	Kaempferol
KoCVAM	Korean Center for the Validation of Alternative Methods
LEC	Lowest Effective Concentration
LIN	Linuron
LTX	L-thyroxine
Luc	ER responsive reporter gene
M	Molar
MEM	Minimum Essential Medium
MESH	The National Library of Medicine's Medical Subject Heading
MET; meth	Methoxychlor
MIF	Mifepristone
MMTV	Mouse Mammary Tumor Virus

MOR	Morin
MPA	Medroxyprogesterone acetate
MSDS	Material Safety Data Sheet
MT	Metallothionein
MTD	Maximum Tolerated Dose
MTEST	Methyl testosterone
na	not available
nc	not calculated
NCGC	NIH Chemical Genomics Center
NEG	Negative
NEN	Norethynodrel
NIL	Nilutamide
NICEATM	U.S. National Toxicology Program Center for the Evaluation of Alternative Toxicological Methods
NIEHS	U.S. National Institute of Environmental Health Sciences
NIH	U.S. National Institutes of Health
NIHS	Japanese National Institutes of Health
NLM	National Library of Medicine
NON	Nonylphenol
Nonylphenol	*p*-n-nonylphenol
NORT	19-nortestosterone
nt	not tested
NTP	National Toxicology Program
NTPSI	National Toxicology Program Substances Inventory
OCT	4-*tert*-octylphenol
OECD	Organisation for Economic Co-operation and Development

OHAN	4-hydroxyandrostenedione
OHTAM	4-hydroxytamoxifen
o,p'-DDT	1,1,1-Trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane
OPPTS	Office of Prevention, Pesticides and Toxic Substances
OX	Oxazepam
Panel	The 24-member scientific expert panel convened in May 2002 to review the information and recommendation provided in the four NICEATM draft BRDs (ER and AR Binding and Transcriptional Activation).
PBARB	Phenobarbital
PBS	Phosphate Buffered Saline
PCY	Procymidone
PN	Presumed Negative
POS	Positive
p,p'-DDE	Dichlorodiphenyldichloroethylene
ppb	Parts per billion. One part in 10^9 molecules.
ppq	Parts per quadrillion. One part in 10^{15} molecules.
PPTH	Phenolphthalin
PROG	Progesterone
PP	Presumed Positive
PRP	Reer Review Panel
PTU	Propylthiouracil
PZE	Pimozide
QA	Quality Assurance
Ral	Raloxifene
REACH	Registration, Evaluation and Authorisation of Chemicals
RES	Resveratrol
RLU	Relative Light Units

RPMI-1640 medium Roswell Park Memorial Institute cell culture medium

RSP	Reserpine
RUC	Rat Uterine Cytosol
SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
SAZ	Sodium azide
SBP	2-*sec*-butylphenol
SD	Standard Deviation
SEM	Standard Error of the Mean
SERM	Selective Estrogen Receptor Modulator
SMT	Study Management Team
SOP	Standard Operating Procedure
SOW	Statement of Work
SPIR	Spironolactone
Std Dev	Standard Deviation
STTA	Stably Transfected Human Estrogen Receptor-α Transcriptional Activation
TA	Transcriptional Activation
TAM	Tamoxifen
TCPA	2,4,5-trichlorophenoxyacetic acid
TEST	Testosterone
TG	Test Guideline
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TREN	17ß-trenbolone
TSH	Thyroid Stimulating Hormone
WHO	World Health Organization
XDS	Xenobiotic Detection Systems, Inc.
VC	Vehicle Control

VIN Vinclozolin

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PREFACE

Endocrine disruptors (EDs) are natural and man-made substances in the environment that interfere with the normal function of hormones in the endocrine system. Public health concerns have resulted largely from studies indicating that animal populations exposed to high levels of these substances have an increased incidence of reproductive and developmental abnormalities (EPA 1997: NAS 1999). In response to growing concerns about possible adverse health effects in humans exposed to such substances, the U.S. Congress enacted relevant provisions to safeguard public health in the Food Quality Protection Act (FQPA) of 1996 (Public Law [P.L.] 104-170) and the 1996 Amendments to the Safe Drinking Water Act (SDWA) (P.L. 104-182). These laws require the U.S. Environmental Protection Agency (EPA) to develop and validate a screening and testing program to identify substances with endocrine disrupting activity. The EPA subsequently proposed an Endocrine Disruptor Screening Program (EDSP) (EPA 1998) and initiated efforts to standardize and validate test methods for inclusion in the EDSP. Validation is necessary to assess the usefulness and limitations of a test method for a specific proposed purpose, and to characterize the extent that test methods are sufficiently accurate and reproducible for their intended use (ICCVAM, 1997).

In April 2000, the U.S. Environmental Protection Agency (EPA) nominated four types of *in vitro* test methods for detecting substances with potential endocrine disrupting activity for review by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). These included *in vitro* ER and AR binding and ER and AR TA test methods (EPA 2001; NIEHS 2001). The EPA also asked ICCVAM to develop performance standards that could be used to define acceptable *in vitro* ER and AR binding and TA assays. It was envisioned that these standards would be based on the performance of adequately validated *in vitro* ER- and AR-based assays.

The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) subsequently prepared Background Review Documents that included all available information on each of the four types of test methods. In a public meeting, the independent international expert panel (Panel) reviewed the information on the 137 assays identified in the BRD and concluded that there were no

adequately validated in vitro ER- or AR-based test methods. Based on recommendations from the Panel, ICCVAM published a list of chemicals that should be used for validation of each of the four types of in vitro test methods, and essential test method components that should be included in each of the standardized test method protocols used for future validation studies (ICCVAM, 2003). ICCVAM recommended that the future performance criteria for performance standards for these methods should be based on test methods that have undergone adequate validation studies using the recommended validation chemicals and essential test method components.

This document provides proposed performance standards based on the results for a test method that has now undergone an independent international validation study. This test method, the LUMI-CELL® BG1Luc4E2 ER TA Test Method (hereafter, BG1Luc ER TA test method) was nominated for validation study by Xenobiotics Detection Systems, Inc. (XDS, Durham, NC). ICCVAM and the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) recommended that the BG1Luc ER TA should be considered a high priority for interlaboratory validation studies based on the lack of adequately validated test methods and the regulatory and public health need for such test methods. NICEATM subsequently led and coordinated an international validation study with its counterparts in Japan (JaCVAM) and Europe (ECVAM) using laboratories sponsored by each validation organization. NICEATM organized a validation Study Management Team (SMT) to oversee the scientific aspects of the validation study and coordinated the day-to-day activities among the participating laboratories. A representative from the recently established Korean Center for the Validation of Alternative Methods (KoCVAM) joined the SMT in 2010.

Based on the results of this study, ICCVAM is now reviewing the validation status of this test method for identification of substances with *in vitro* ER agonist or antagonist activity. NICEATM and the ICCVAM Interagency Endocrine Disruptors Working Group (EDWG) prepared a draft BRD that provides a comprehensive description and the data from the validation study used to assess the accuracy and reliability of the BG1Luc ER TA test method (ICCVAM, 2011a).

NICEATM will convene an international independent scientific peer review panel (Panel) that will meet in public on March 29-30, 2011. The Panel is charged with reviewing the draft BRD for completeness, assessing the extent that established validation and acceptance criteria have been adequately addressed, and determining the extent that the data and information support draft ICCVAM test method recommendations on the usefulness and limitations for the BG1Luc ER TA test method. The Panel will also evaluate these proposed performance standards.

The Panel includes expert scientists nominated by ECVAM, JaCVAM, and KoCVAM. ICCVAM will consider the conclusions and recommendations of the Panel, along with comments from the public and SACATM, and then finalize the BRD and test method recommendations. These will be forwarded to Federal agencies for their consideration and acceptance decisions where appropriate. The BG1Luc ER TA test method protocol and performance standards will also be forwarded to the OECD Test Guidelines Programme for consideration and adoption as international testing guidelines.

We gratefully acknowledge the organizations and scientists who generated and provided data and information for this document, especially the staff at the participating validation laboratories at XDS, Inc in Durham, North Carolina, Hyoshi Inc in Japan, and the In Vitro Methods Unit at ECVAM in Italy. We would also like to recognize the efforts of the individuals who contributed to its preparation, review, and revision. We thank Dr. David Hattan (U.S. Food and Drug Administration) for serving as Chair of the EDWG, as well as the members of the EDWG and ICCVAM representatives who subsequently reviewed and provided comments throughout the process leading to this draft BRD. We also want to thank Dr. Warren Casey, Deputy Director of NICEATM, for his excellent leadership and extensive efforts on this project.

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EXECUTIVE SUMMARY

Background

In April 2000, the U.S. Environmental Protection Agency (EPA) nominated four types of *in vitro* test methods for detecting substances with potential to interfere with the normal function of hormones in the endocrine system (i.e., endocrine disruptors [EDs]) (EPA 2001; NIEHS 2001) for review by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). ICCVAM subsequently recommended that these methods should undergo independent scientific peer review based on their potential interagency applicability and public health significance. The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) compiled available data and information on the four types of test methods (*in vitro* ER and AR binding and transcriptional activation [TA] test methods). ICCVAM, the ICCVAM Interagency Endocrine Disruptor Working Group (EDWG), and NICEATM prepared four background review documents (BRDs) that detailed the available data and information needed to evaluate the current validation status of each of the four types of test methods.

In collaboration with ICCVAM and the EDWG, NICEATM organized an independent evaluation of these *in vitro* test methods. ICCVAM considered the Panel's conclusions and recommendations and public comments. ICCVAM then developed test method recommendations that included minimum procedural standards and a list of 78 reference substances that should be used to standardize and validate *in vitro* ER and AR binding and TA test methods (ICCVAM 2003).

In January 2004, Xenobiotics Detection Systems, Inc. (XDS, Durham, NC) nominated their LUMI-CELL® BG1Luc4E2 ER TA Test Method (hereafter, BG1 Luc ER TA) for an interlaboratory validation study. This method uses BG-1 cells (a human ovarian carcinoma cell line) that are stably transfected with an estrogen-responsive luciferase reporter gene to measure whether and to what extent a substance induces or inhibits TA activity via ER mediated pathways (Denison and Heath-Pagliuso 1998). Included in the nomination package were test results from XDS for 56 of the 78 ICCVAM Reference Substances for agonist activity and 16 of the 78 ICCVAM Reference Substances for antagonist activity. These studies were funded primarily by a Small Business Innovation Research (SBIR) grant

(SBIR43ES010533-01) from the National Institute of Environmental Health Sciences (NIEHS).

The BG1Luc ER TA was considered by ICCVAM as a high priority for interlaboratory validation studies and the NIEHS agreed to support this effort. NICEATM led and coordinated an international interlaboratory validation study with its counterparts at the Japanese Center for the Validation of Alternative Methods and the Europe Centre for the Validation of Alternative Methods. The BG1Luc ER TA was evaluated in four phases, during which the 78 ICCVAM Recommended Substances were tested, using laboratories in the U.S. (XDS), Europe (ECVAM), and Japan (Hiyoshi Corporation [Hiyoshi]).

NICEATM, in conjunction with the EDWG prepared this draft BRD that summarizes the available data and information regarding the current validation status of the BG1 Luc ER TA test method.

BG1Luc ER TA Test Method Protocol

The BG1Luc ER TA utilizes an ER responsive reporter gene (*luc*) in the human ovarian adenocarcinoma cell line, BG-1, to detect substances with *in vitro* ER agonist or antagonist activity. An assessment of cell viability, to help define the upper limit for test substance concentrations, is performed using visual observation of cell density and morphology to assign cell viability scores. ER-mediated transcription of the *luc* gene results in the production of luciferase, the activity of which is quantified using a luminometer. In accordance with earlier ICCVAM recommendations, 17β-estradiol (E2, CASRN 50-28-2) is used as the reference estrogen to demonstrate the adequacy of the ER TA test method. Raloxifene is utilized as a reference standard in the ER TA antagonist test method. A concentration-response curve can be established to provide qualitative and quantitative information regarding the *in vitro* estrogenic activity of a test substance. The advantages of using a luciferase reporter gene system are low background, high sensitivity, rapidity, and a wide dynamic range.

Substances used in the Validation Study

The ICCVAM list of 78 recommended reference substance list was developed, based on a review of the literature, to assess test method performance of four different assays (ER TA and AR TA agonist and antagonist assays). Only those substances that could be definitively

classified as positive or negative for ER TA activity (48 unique substances) were used to assess accuracy. Separate lists were generated for evaluating test method accuracy for agonist (42 substances; 33 Positive, 9 Negative) and antagonist (25 substances; 3 Positive, 22 Negative) activity.

BG1Luc ER TA Test Method Accuracy

The BG1Luc ER TA was evaluated for its ability to correctly identify estrogen receptor agonists and antagonists. For this analysis, test substance classification (positive or negative for ER agonist/antagonist activity) obtained during the validation study was compared to the classification of the same substance based on a preponderance of published data. Positive or negative classifications of BG1Luc ER TA data were based on the majority classification assigned using results from each of the three participating laboratories. Test method accuracy was evaluated based on a number of analyses, but the primary evaluation of the BG1 Luc ER TA is based on two comparisons: 1) the extent to which the BG1 Luc ER TA result corresponds to the ICCVAM reference classification for each substance, and 2) the comparative accuracy of the BG1 Luc ER TA and the CERI STTA (OECD, 2009).

Of the 42 substances used to evaluate agonist accuracy, 7 (17%) had "inadequate" testing results in the BG1 Luc ER TA and were therefore excluded from the analysis, leaving 35 (28 Positive, 7 Negative) substances for evaluation. The BG1 Luc ER TA produced the following results when compared to the reference classifications for these 35 substances: concordance of 97% (34/35), sensitivity of 96% (27/28), specificity of 100% (7/7), a false positive rate of 0% (0/7), and a false negative rate of 4% (1/28).

The CERI STTA is the only ER TA test method currently accepted by U.S. regulatory agencies for ER agonist testing[1]. When using the 26 reference substances for which both BG1 Luc ER TA and CERI STTA data are available, identical accuracy statistics are calculated: concordance of 96% (25/26), sensitivity of 95% (21/22), specificity of 100% (4/4), a false positive rate of 0% (0/4), and a false negative rate of 5% (1/22).

All 25 of the antagonist reference substances produced a definitive result in the BG1 Luc ER TA and yielded an overall concordance of 100% (25/25), sensitivity of 100% (3/3),

[1] Currently, there are no ER antagonist test methods that are accepted by U.S. regulatory agencies.

specificity of 100% (22/22), a false positive rate of 0% (0/22), and a false negative rate of 0% (0/3).

Although the primary goal of the BG1Luc ER TA is to provide a qualitative assessment of estrogenic/anti-estrogenic activity, quantitative measures of activity (i.e., EC_{50} and IC_{50} values) are usually obtained for positive results. EC_{50} and IC_{50} values obtained from BG1Luc ER TA test results were compared to median values from other ER TA test methods reported in the literature and this comparison produced a high correlation. BG1Luc ER TA test results were also examined for concordance with published reports of ER binding and there was 97% (33/34) concordance between the BG1Luc ER TA and ER binding data from the literature. The only discordant substance was positive in BG1 Luc ER TA and negative based on ER binding data. Similarly, based on a comparison with available data in the *in vivo* uterotrophic assay, 13 substances with conclusive test results in the BG1Luc ER TA agonist test method produced overall concordance of 92% (12/13). The only discordant substance was positive in BG1 Luc ER TA and negative based on uterotrophic data.

BG1Luc ER TA Test Method Reliability

Intralaboratory Reproducibility

Intralaboratory reproducibility of the BG1Luc4E2 agonist and antagonist test methods was assessed by comparing: 1) reference standard and control results for all plates tested within each laboratory during the course of the validation study and 2) results from Phase 2a and 2b testing during which 12 substances were tested in at least three independent experiments in each of the three laboratories.

In the agonist test method, mean fold induction in each lab ranged from 4.6 to 7.8 fold and E2 reference standard EC_{50} values ranged between 8.0×10^{-12} to 1.2×10^{-11} M.

The resulting classifications for each of the 12 substances that were tested at least three times at each laboratory were used to evaluate the extent of intralaboratory agreement. Although the classifications for some of the test substances differed among the laboratories, there was 100% agreement within each laboratory for each of the three repeat tests.

In the antagonist testing, mean fold reduction ranged from 8.0 to 9.9 fold and Raloxifene reference standard IC_{50} values ranged between 1.1×10^{-9} to 1.3×10^{-9} M.

The classifications for each of the 12 substances that were tested at least three times at each laboratory were used to evaluate the extent of intralaboratory agreement. Although the classifications for some of the test substances differed among the laboratories, there was 100% agreement within each laboratory for each of the three repeat tests.

Interlaboratory Reproducibility

Interlaboratory reproducibility was determined for the 12 substances that were tested at least three times for agonist and antagonist activity during Phase 2, at each of the three laboratories. The classifications for each of the 41 substances that were tested once for agonist and antagonist activity at all three laboratories during Phase 3 were also used to evaluate the extent of interlaboratory agreement.

For each of the 12 substances that were tested at least three times for agonist and antagonist activity during Phase 2, agreement among the three laboratories was determined based on the consensus classification assigned by each laboratory for each of the 12 substances. The three laboratories agreed on 67% (8/12) of the substances tested for agonist activity. Among the substances tested for antagonist activity, there was 100% agreement among the three laboratories for all 12 substances.

The classifications for each of the 41 substances that were tested once for agonist activity at all three laboratories during Phase 3 were also used to evaluate the extent of interlaboratory agreement. Unlike Phase 2, some of the substances tested in Phase 3 produced inadequate results. Of the 41 substances tested in Phase 3, 88% (36/41) produced a definitive result in at least two laboratories, and were therefore used for the assessment of reproducibility. Among these 36 substances, the three laboratories agreed on 83% (30/36) of the substances tested for agonist activity. Among substances tested for antagonist activity, definitive results were produced for all substances and the three laboratories agreed on 93% (38/41) of the substances tested.

Animal Welfare Considerations

The BG1Luc ER TA may be applicable for addressing the ER TA component of the EPA EDSP Tier 1 screening battery. Although the EDSP currently includes an *in vitro* ER TA test method for ER agonist testing (i.e., the CERI STTA method), there currently are no *in vitro* test methods accepted for ER antagonist testing. Therefore, the BG1Luc ER TA provides an

opportunity to reduce animal use in ED testing by identifying substances that may either enhance and/or inhibit the activation of the ER. This information can be used as part of a weight-of-evidence approach to prioritize substances for additional investigation of ED activity in test methods that require animals.

There are currently three *in vivo* methods commonly used by regulators to assess the estrogenic potential of substances: rat uterotrophic, rat pubertal female, and fish short-term reproduction assay. In addition, the "*in vitro*" Rat Uterine Cytosol ER binding assay also requires the use of animals as a source of ER. Although the BG1Luc ER TA is not propose as a direct replacement for any of these existing methods, it could be incorporated as part of a weight of evidence approach to reduce or eliminate the need for the use of animals for identifying substances with potential estrogenic or anti-estrogenic activity.

Test Method Transferability

Transferability of the BG1Luc ER TA was demonstrated based on results of the interlaboratory validation study that are detailed above. The primary practical considerations associated with the BG1Luc ER TA are the availability of the requisite cell line and the standard laboratory equipment necessary to conduct sterile cell culture procedures. The BG-1Luc4E2 cell line is available upon request from Dr. Michael S. Denison, Department of Environmental Toxicology, University of California, Davis. The level of training, expertise, and time needed to conduct the BG1Luc ER TA should be similar to the currently accepted CERI STTA method.

Draft ICCVAM Test Method Recommendations

After considering the data and analysis provided in this background review document, ICCVAM developed draft recommendations on the usefulness and limitations of the BG1Luc ER TA test method as a screening test to identify substances with estrogen agonist activity. ICCVAM also developed draft recommendations for a standardized test method protocol, proposed future studies, and performance standards. These are provided in a separate document, *Draft ICCVAM Test Method Recommendations: The BG1 Luc ER TA Test Method.*